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Expression of simian immunodeficiency virus Nef protein in CD4$^+$ T cells leads to a molecular profile of viral persistence and immune evasion

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Abstract

The Nef protein of human immunodeficiency virus and simian immunodeficiency virus is expressed early in infection and plays an important role in disease progression in vivo. In addition, Nef has been shown to modulate cellular functions. To decipher Nef-mediated changes in gene expression, we utilized DNA microarray analysis to elucidate changes in gene expression in a Jurkat CD4$^+$ T-cell line stably expressing SIV-Nef protein under the control of an inducible promoter. Our results showed that genes associated with antigen presentation including members of the T-cell receptor and major histocompatibility class 1 complex were consistently down-regulated at the transcript level in SIV-Nef-expressing cells. In addition, Nef induced a transcriptional profile of cell-cycle-related genes that support the survival of Nef-expressing cells. Furthermore, Nef enhanced the transcription of genes encoding enzymes and factors that catalyze the biosynthesis of membrane glycolipids and phospholipids. In conclusion, gene expression profiling showed that SIV-Nef induces a transcriptional profile in CD4$^+$ T cells that promotes immune evasion and cell survival, thus facilitating viral persistence.

Keywords: SIV-Nef; Immune evasion; DNA microarray; Antigen presentation; Viral persistence

Introduction

Retroviruses, including human and simian immunodeficiency viruses (HIV and SIV), have evolved elaborate strategies to evade host immune surveillance in order to replicate and establish a persistent infection. One of the strategies employed by HIV and SIV is the impairment of antigen presentation by down-regulation of host MHC molecules. In addition, HIV infection subverts the apoptotic machinery in both infected and uninfected cells, thereby facilitating the survival of virally infected cells. Among the viral accessory proteins, Nef has been shown to contribute to immune evasion. The *nef* gene of HIV and SIV encodes 25- to 27-kDa myristoylated protein that is expressed early during the virus life cycle and plays a crucial role in disease progression (Deacon et al., 1995; Kestler et al., 1991; Kirchhoff et al., 1995). Multiple cellular functions have been attributed to Nef protein. Down-regulation of CD4 molecules from the cell surface is the most clearly documented function of Nef involving direct bridging of the cytoplasmic tail of CD4 to an adaptor protein which targets it for endocytosis and degradation (Aiken et al., 1994; Bandres et al., 1995; Guy et al., 1987; Lama et al., 1999; Mariani and Skowronski, 1993; Rhee and Marsh, 1994). The down-regulation of CD4 molecules is thought to facilitate virion release (Ross et al., 1999). Nef is also known to modulate T-cell activation through the interaction between Nef and signal transduction proteins (Baur et al., 1994; Sawai et al., 1994; Skowronski et al., 1993).

The most pathophysiologically significant effect of Nef on CD4$^+$ T cells expressing Nef and virally infected cells is the evasion of immune responses. Nef down-regulates major histocompatibility complex class I (MHC-I) molecules from the cell surface (Greenberg et al., 1998; Schwartz et al., 1996; Swigut et al., 2000). This function of Nef involves indirect interaction with the cytoplasmic tail of MHC-I A and B molecules leading to endocytosis and confers partial protection from recognition and lysis by cytotoxic T cells (Collins et al., 1998; Greenberg et al., 1998; Schwartz et al., 1996). Nef has also been shown to enhance virus replication and infectivity through a mechanism that involves enhanced biosynthesis and transportation of cholesterol

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and ganglioside to lipid rafts (Zheng et al., 2003). Lipid rafts are microdomains in the plasma membrane enriched in sphingolipids and cholesterol that are essential for egress from and entry of HIV into target cells (Zheng et al., 2001, 2003).

Comparison of SIV and HIV-1 Nef has revealed similarities and differences in function and structure between these proteins (Piguet and Trono, 1999). Both SIV and HIV-1 Nef show similar in vitro activities: down-regulation of CD4 and MHC-I cell surface expression, enhancement of virion infectivity, stimulation of viral replication in primary lymphocytes, modulation of T-cell signaling pathway and interaction with cellular serine/threonine and tyrosine kinases. The Nef proteins of both viruses contain a myristylated N-terminal and show homology, mainly in the central domain (Renkema and Saksela, 2000; Shugars et al., 1993). Despite these similarities, some noteworthy differences exist between SIV and HIV-1 Nef. In contrast to HIV-1 Nef, SIV-Nef contains additional amino acids residues at the N-terminus that are absent in HIV-1 Nef. Moreover, SIV and HIV-1 Nef interact with cellular proteins through distinct motifs and may utilize overlapping but distinct target sites for CD4 down-regulation (Bresnahan et al., 1998; Craig et al., 1998; Greenberg et al., 1998; Hua and Cullen, 1997; Lock et al., 1999; Piguet et al., 1998). HIV-1 Nef contains three repeats of the putative SH3 binding domain (PxP) important for the binding of Src family tyrosine kinases. In contrast, SIV-Nef contains only one corresponding PxP motif (Arold et al., 1997; Lee et al., 1995; Saksela et al., 1995). Thus, SIV-Nef and HIV-1 Nef are functionally similar but utilize different mechanisms to carry out similar functions (Bresnahan et al., 1998; Greenway et al., 1999; Hua and Cullen, 1997; Iafrate et al., 1997; Lang et al., 1997; Lock et al., 1999; Piguet et al., 1998).

SHIV nef chimeric viruses, constructed by replacing the SIVmac 239 *nef* gene with HIV-1 nef in a SIVmac 239 backbone, provided a valuable tool for investigating the interchangeability of SIV and HIV-1 Nef protein functions (Kirchhoff et al., 1999; Mandell et al., 1999; Shibata et al., 1991, 1997; Sinclair et al., 1997). These studies demonstrated that HIV-Nef expression in macaque cells exerted similar cellular effects and that HIV Nef and SIV-Nef are functionally interchangeable. Rhesus macaques experimentally infected with SHIV nef clones revealed that HIV Nef induces an AIDS-like disease in macaques. While the functional equivalence between HIV-1 and SIV-Nef has been demonstrated in vitro and in vivo, the suitability of the SHIV/macaque model for the study of the role of HIV-1 Nef in viral pathogenicity is yet to be determined.

Previous work investigating the mechanisms by which Nef exerts its effects on $CD4^+$ T cells has focused on delineating posttranscriptional interactions between Nef and cellular signaling and trafficking proteins (Piguet and Trono, 1999). However, effects of Nef on the transcription of genes involved in immune evasion and viral persistence have not been fully investigated. Given the complexity of pathways mediating these cellular processes and the paucity of information on the underlying mechanisms of Nef effects on T cells, we elected to characterize global gene expression profiles in Nef-expressing $CD4^+$ T cells using DNA microarray analysis. The effects of Nef on the transcription of cellular genes in different cell types of epithelial, neural and lymphocytes origin were previously investigated using DNA microarray analysis (Kramer-Hammerle et al., 2005; Shaheduzzaman et al., 2002; Simmons et al., 2001; van't Wout et al., 2005). These studies reported that Nef induced a transcription profile that enhanced cell activation and increased expression of genes that regulate apoptosis, biosynthesis and transport of membrane lipids. However, due to differences in cell type, the timing and the levels of Nef expressed, relevance of the processes in Nef-expressing cells during viral infection has been difficult to ascertain. We developed a Jurkat T-cell line that stably expresses SIV-Nef protein under the control of an inducible promoter (Ndolo et al., 2002). Human T-cell lines have been extensively used for the expression of SIV-Nef by other investigators and it was shown that SIV-Nef exerted similar effects as HIV-1 Nef expressed in human cells (Alexander et al., 1999; Baur et al., 1994; Sawai et al., 1995; Walk et al., 2001; Yoon et al., 2001). Similar Nef activities have also been reported in primary rhesus macaques CD4 T cells infected with SHIV nef recombinant viruses (Kirchhoff et al., 1999; Mandell et al., 1999; Shibata et al., 1997; Sinclair et al., 1997). We show that Nef mediates the down-modulation of the transcription of genes belonging to members of the MHC-I complex that mediate antigen presentation, thus facilitating immune evasion. Previous studies focused on endocytotic mechanisms. In addition, SIV-Nef enhances the transcription of genes encoding enzymes and factors that catalyze the biosynthesis of membrane lipids, facilitating the formation of lipid rafts that are pivotal for enhanced viral infectivity. Our data are in agreement with recently reported HIV Nef-mediated up-regulation in T cells of genes encoding enzymes involved in RNA processing, protein synthesis and cholesterol biosynthesis (Shaheduzzaman et al., 2002). Overall, our study indicates that SIV-Nef induces a transcriptional profile in $CD4^+$ T cells that promotes immune evasion and cell survival, thus facilitating viral persistence.

Results

Ecdysone-inducible expression of SIV-Nef

Previous attempts to stably express Nef in T cells have been hampered by the fact that long-term expression of Nef is toxic and results in selection of truncated Nef protein and eventual loss of expression (Baur et al., 1997; Baur et al., 1994; Walk et al., 2001). To overcome these limitations, we used the ecdysone-inducible expression system to achieve regulated expression of SIV-Nef in Jurkat T cells. A low basal level of Nef expression was observed in Jurkat T cells transfected with the pIND-CD8-nef and pVgRxR plasmids but in the absence of ponasterone A induction. Since leakage proof expression of recombinant proteins is difficult to achieve in inducible protein expression systems, we selected clone B5 which showed a five-fold increase in Nef expression upon induction but had a minimal basal expression in Nef-expressing Jurkat T cells in the absences of ponasterone A induction (Fig. 1). Expression levels of Nef protein at 24 h post-induction were found to be equivalent to levels of Nef

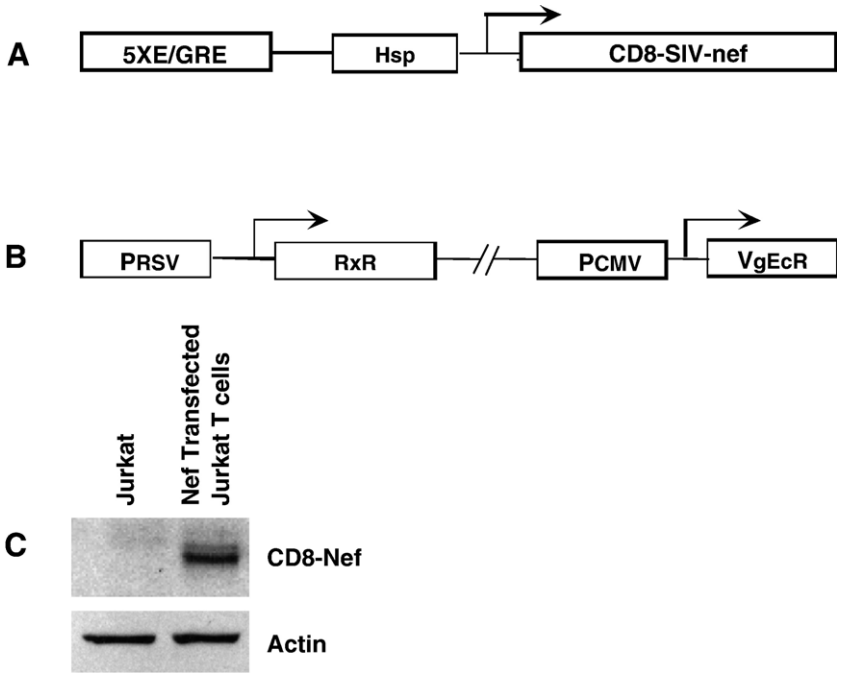

Fig. 1. Inducible expression of SIV-Nef in Jurkat T cells. (A) Schematic diagram of pIND-CD8-Nef construct. (B) Schematic diagram of pVgRxR regulatory plasmid. (C) Stable expression of SIV-Nef in Jurkat T-cell line. Jurkat T cells were stably transfected with plasmids pIND-CD8-Nef encoding CD8-Nef chimera and pVgRxR encoding *Drosophila* ecdysone hormone receptor subunits RxR and EcR. Clones resistant to both G418 and Zeocin were screened for SIV-Nef expression by immunoblotting following induction with ponasterone A. Nef-expressing clone B5 which showed a five-fold increase in Nef expression upon induction and minimal basal expression in comparison to non-ponasterone-A-induced Nef-expressing Jurkat T cells.

protein observed in SIVmac 251 infected primary $CD4^+$ T cells in vivo and CEMx174 cells during early stages of infection (Ndolo et al., 2002; Walk et al., 2001). As expected, Nef expression was not observed in ponasterone-A-induced untransfected Jurkat cells and Jurkat cells stably transfected with control plasmids. We refer to these controls collectively as ponasterone-A-induced non-Nef-expressing Jurkat control T cells in all our subsequent results.

Functional categories of cellular genes modulated by SIV-Nef

We utilized DNA microarray analysis to elucidate changes in gene expression in Jurkat T cells stably expressing SIV-Nef protein. Labeled cRNA was hybridized to Affymetrix U133A GeneChips, and changes in the level of transcription of each gene were determined by statistical analysis of the difference in mean fluorescence between ponasterone-A-induced-Nef-expressing and ponasterone-A-induced non-Nef-expressing Jurkat T cells over three replicate experiments.

In Jurkat cells expressing SIV-Nef, 3542 genes were differentially expressed of which 2392 genes were up-regulated whereas 1232 genes were down-regulated in comparison to ponasterone-A-induced non-Nef-expressing control Jurkat cells. The DNA microarray data have been deposited as supplementary data at the Gene Expression Omnibus database through the NCBI website. These supplementary data are available at the following link (http://www.ncbi.nlm.nih.gov/projects/geo/query/acc.cgi?acc=GSE4785). Genes with increased or decreased expression were statistically analyzed for the presence of underlying biological themes (by the percentage of genes and Fisher exact probability score) as shown in Fig. 2. Genes up-regulated by Nef were predominantly involved in lipid metabolism, chaperone activity, protein targeting and various aspects of RNA metabolism, including mRNA splicing and processing, transcription, translation and degradation. Significantly down-regulated functional categories included defense responses, cell cycle, cell adhesion, cytoskeletal protein binding, gamma-glutamyl transferase and phosphoric ester hyrolase activity and glucose metabolism. The observed gene expression profile modulated by SIV-Nef in $CD4^+$ T cells in our study was similar to that reported on HIV-1 Nef (Kramer-Hammerle et al., 2005; Shaheduzzaman et al., 2002; Simmons et al., 2001; van't Wout et al., 2005).

Expression of antigen processing and presentation genes is repressed by SIV-Nef

Major histocompatibility complex (MHC), class I transcripts were among the group of down-regulated genes involved in defense response (Fig. 2). Transcription of genes associated with antigen presentation, including MHC 1B, IC, IE, IF and IG, was significantly down-regulated (Fig. 3A), however, genes associated with antigen presentation via proteasome degradation were up-regulated in Nef-expressing cells. In addition, expression of both 26S and 20S proteasome subunits, ubiquitin/E2 complex molecules and activators of the proteasome complex was increased, suggesting that degradation of intact proteins and peptides may be up-regulated in Nef-expressing cells (Fig 3B). Previous studies have reported Nef-mediated down-regulation of HLA-A and B allotypes from the cell

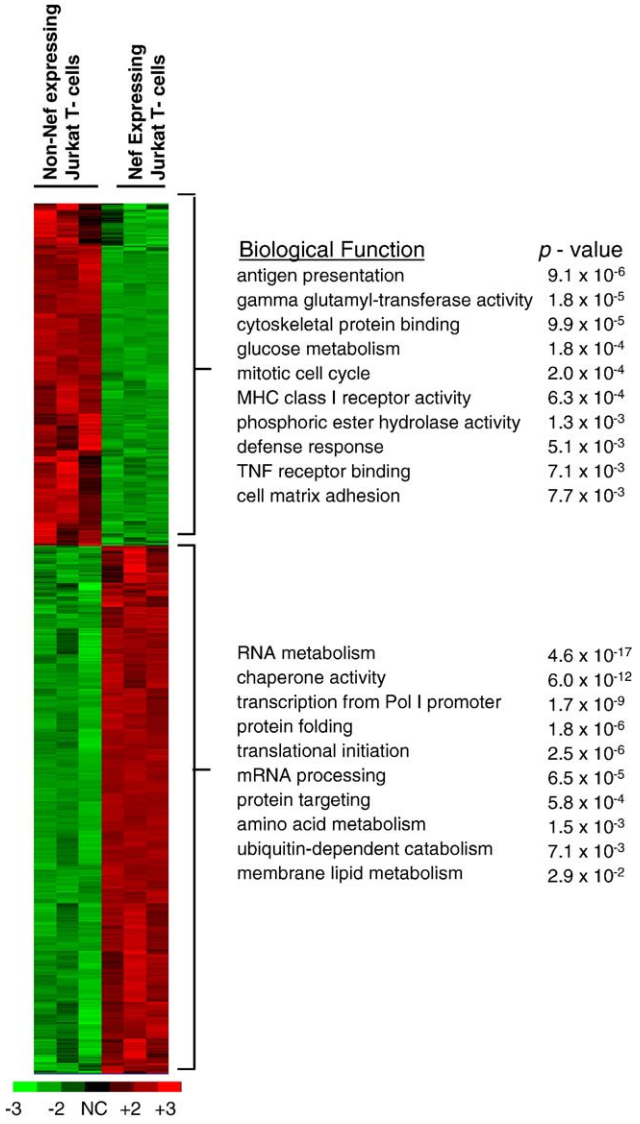

Fig. 2. Hierarchical clustering of genes modulated by SIV-Nef. Hierarchical clustering was used to identify genes differentially modulated by SIV-Nef. Twenty broad functional clusters were identified. Genes involved in RNA metabolism, RNA processing, translation initiation, membrane lipid metabolism and transcription were up-regulated. Genes involved in antigen presentation, mitotic cell cycle, defense response, MHC class I receptor activity and tumor necrosis factor receptor binding were down-regulated. The scale at the bottom indicated the magnitude of up- or down-regulation (fold change in expression). Red bars represent up-regulated while green bars represent down-regulated genes. Black bars represent genes not differentially modulated by SIV-Nef. Genes were selected based on a ≥ 1.5-fold change and a p value of ≤ 0.05.

surface, thus protecting HIV-infected cells from CTL-mediated lysis (Collins et al., 1998; Schwartz et al., 1996; Swigut et al., 2000). Our results demonstrated that suppression of MHC genes at the transcriptional level is also likely to be a key molecular mechanism for Nef-induced immune evasion.

In addition to MHC class I, we found substantial down-regulation of the T-cell receptor alpha (−1.7-fold) and gamma chains (−1.9-fold), SCAP1 (−2.1) and CD69 (−4.6-fold), suggesting a general suppression of transcription of antigen presentation molecules (Fig. 3). CD69 is a type II integral membrane protein that also plays a role in modulation of inflammatory responses and apoptosis (Foerster et al., 2002; Lopez-Cabrera et al., 1993, 1995; Santis et al., 1995; Walsh et al., 1996). Nef-mediated down-regulation of CD69 may suggest a protective role for Nef against Fas-mediated apoptosis of infected cells in addition to evasion from cell-mediated immune responses. SCAP1 encodes an src kinase-associated adaptor phosphoprotein preferentially expressed in T cells (Wang et al., 2003). SCAP1 has been shown to regulate integrin-mediated adhesion and conjugate formation between T cells and antigen-presenting cells and positively regulates TCR-mediated gene transcription (Wu et al., 2002). In the context of known functions of SCAP1, Nef-mediated down-regulation of SCAP1 may be interpreted to play a role in the repression of TCR receptor expression, thus providing additional mechanisms to facilitate immune evasion.

Another category of genes within the immune responses cluster up-regulated in SIV-Nef-expressing Jurkat T cells included CD59 (+2.0-fold change; $p<1e{-}07$), CD1D (4.3-fold change; $p<1e{-}07$) and G1P2 (+2.4-fold change; $p<1e{-}07$). CD59 is a membrane protein that regulates complement and inhibits the formation of membrane attack complex pores on the surface of CD4 T cells (Rollins and Sims, 1990). Nef-mediated up-regulation of CD59 expression may protect SIV-Nef-expressing cells from complement-mediated cell lysis. CD1 genes encode a family of glycoproteins that are related to MHC molecules and are involved in the presentation of non-peptide antigens to T cells (Porcelli and Modlin, 1999). CD1D genes belong to group II, CD1d molecules present α-galactosyl-ceramide to NK T cells (Kawano et al., 1997). Interestingly, it has been shown that expression of CD1 molecules protects target cells from NK-cell-mediated lysis, suggesting that Nef-induced up-regulation of CD1D transcripts in $CD4^+$ T cells may also serve to protect Nef-expressing cells from NK-cell-mediated killing (Campos-Martin et al., 2004; Chang et al., 1999). G1P2 is a member of IFN-stimulated genes (ISGs) and encodes a ubiquitin-like protein that posttranslationally modifies cellular proteins by forming covalent conjugates in response to IFN and other markers of viral infection (Haas et al., 1987). Overall, these data indicate that SIV-Nef mediated down-modulation at the transcriptional level of factors involved in antigen presentation and up-regulation of factors that protect cells from CD8 T cells and NK-mediated cytolysis provides multiple mechanisms for immune evasion during viral infection.

SIV-Nef mediated modulation of cell cycle regulators promotes cell survival and viral replication

SIV-Nef-expressing Jurkat T cells also displayed a complex dysregulation of genes encoding for proteins involved in cell cycle regulation. Eight of the ten genes modulated by Nef in this functional cluster were down-regulated including cyclins G_1 and G_2, CHFR, CDK6, HK2, HDAC9, CDC25C, CLK3, CCNB1P1, CDC2L6, CD40LG and other cell-cycle-related genes (Fig. 4). The protein encoded by CDK6 is a catalytic subunit of the protein kinase complex that is important for cell cycle G_1 phase progression and G_1/S transition (Meyerson and Harlow, 1994). CDC25C encodes a cell cycle regulatory protein belonging to Cdc25 tyrosine phosphatase family (Sadhu et al.,

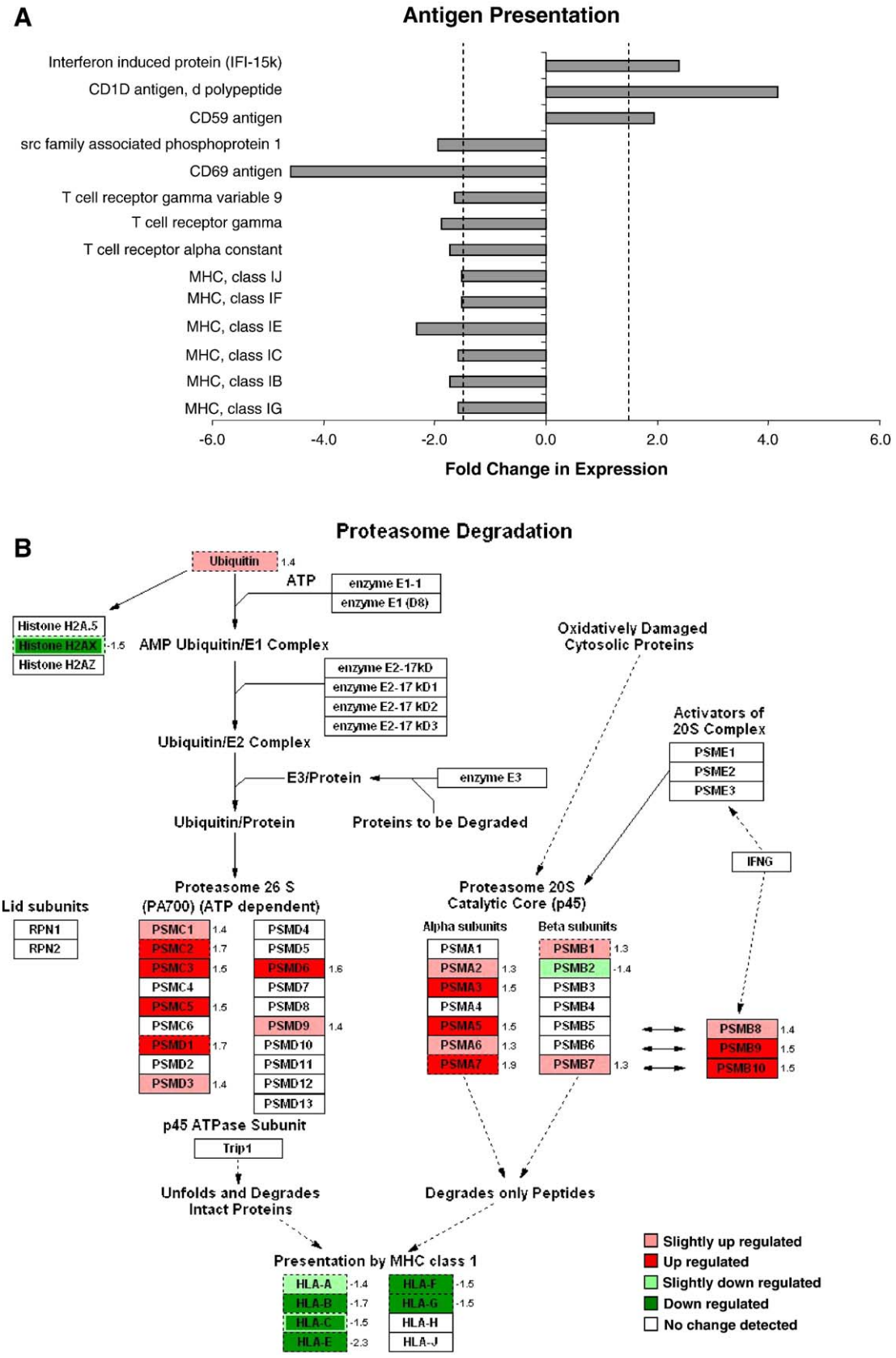

Fig. 3. Antigen-presentation-associated genes modulated by SIV-Nef. (A) SIV-Nef-expressing Jurkat T cells showed between −5.0 and −1.5-fold down-regulation in the transcript levels of MHC class I, TCR genes involved in antigen presentation and recognition. (B) Expression of proteasome degradation-related genes, 26S and 20S proteasome subunits and ubiquitin/E2 complex molecules is enhanced in SIV-Nef-expressing Jurkat T cells.

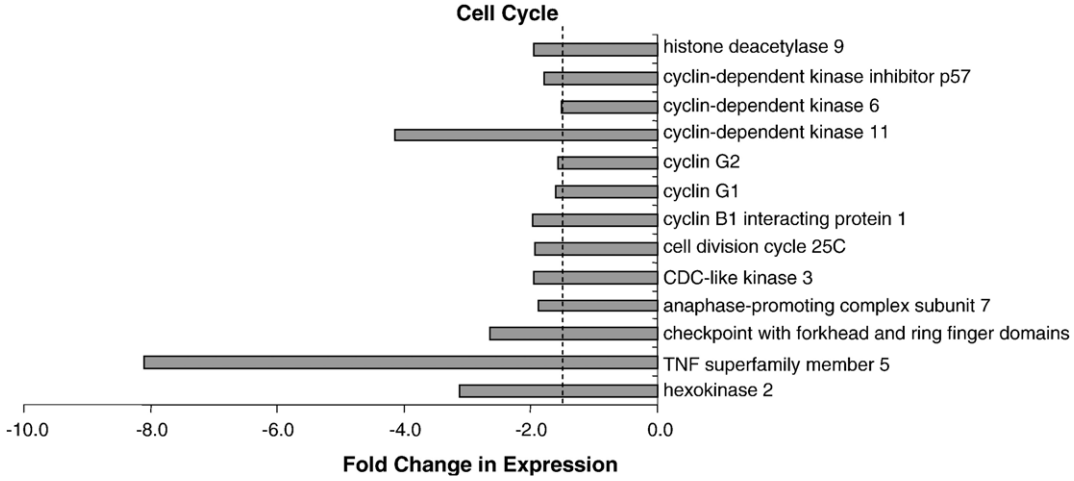

Fig. 4. Cell-cycle-related gene modulated by SIV-Nef expression. Genes involved in the regulation of the cell cycle such as cyclins and cyclin-dependent kinases were significantly down-regulated in SIV-Nef-expressing Jurkat T cells. Magnitude of down-regulation in fold change expression is indicated on the *x* axis.

1990). CDC25C mediates the dephosphorylation of cyclin-B-bound CDC2 and triggers entry into mitosis (Strausfeld et al., 1991). CCNB1IP1 encodes for a member of the E3 ubiquitin ligase family of proteins and modulates cell cycle progression through G_2/M checkpoint (Toby et al., 2003). Down-regulation of this gene by Nef may delay the entry of cells into mitosis, thus facilitating the completion of the transcription of viral genes and viral replication. HDAC9 encodes a histone deacetylase. Histone acetylation and deacetylation play essential roles in modifying chromatin structure and regulating gene expression. HDAC9 has been implicated in modulating the transcriptional repression of gene expression during cell activation and differentiation, development of inflammation and cell cycle regulation (Shaffer et al., 2000). Nef-induced down-regulation of HDAC9 may overcome this repression, facilitating T-cell activation (Esau et al., 2001). CLK3 and CHFR encode for protein kinases that regulate the transition of cells through G_2/M phase cell cycle checkpoint control (Hanes et al., 1994; Matsusaka and Pines, 2004). The down-regulation of these regulators by Nef may overcome the G_2/M block and exit from the cell cycle, thus contributing to cell survival and viral replication. CD40LG encodes for the CD154 (CD40− ligand) which is expressed on the surface of T cells. CD154 regulates B cell functions by engaging CD40 on B cell surface (Xu and Song, 2004).

In contrast to the down-regulation of cell-cycle-associated genes, we found that STAG1 and SKB1 genes were up-regulated by SIV-Nef (+2.1 and +2.0-fold change respectively). SKB1 encodes a protein that negatively regulates mitosis (Gilbreth et al., 1998). Over-expression of SKB1 has been shown to restore cell morphology and viability in cells whose transition from G_2 to M phase of the cell division cycle has been blocked, thus inhibiting the exit of cell from G_2 phase (Okayama et al., 1996). STAG1 encodes a subunit of the cohesin complex, which is required for sister chromatids cohesion during mitosis and meiosis (Anazawa et al., 2004). Expression of STAG1 is essential for cell viability (Haering et al., 2002). Down-regulation of STAG1 and SKB1 transcripts by SIV-Nef may mediate cell cycle transition at G_1/S or G_2/M check points. Overall, these data suggest that Nef induces a transcriptional profile of cell-cycle-related genes that supports survival of Nef-expressing cells by enhancing their transition through cell cycle checkpoint controls.

Increased expression of gene regulating RNA processing, protein biosynthesis and degradation in SIV-Nef-expressing cells

Annotation of genes modulated by Nef showed that fifty four genes involved in RNA processing, protein biosynthesis and degradation were up-regulated in SIV-Nef-expressing T cells (Table 1). Of the fifty four up-regulated genes, six encode proteins involved in RNA splicing (SFRS3, SFRS6, SFRS10, SFPQ, POP1, TSEN2), four encode translation factors (EIF5, ETF1, EIF1AX, EIF1AY), three encode mitochondrial ribosomal proteins (MRPL1, MRPL14, MRPL19), four encode for protease (IMMP1L, ZMPSTE24, ADAM10, DF, TFRC) and thirteen encode for transcription factors or protein that interact with RNA polymerase. HIV-1 Nef has been reported to up-regulate genes involved in RNA metabolism such as Topoisomerase 1, RNA pol II subunits, ElonginA, IPP isomerase, Crystalin zeta and protein tyrosine kinase 9 in HeLa cells (Shaheduzzaman et al., 2002). Up-regulation of these factors may facilitate the production of viral messages required at later stages of the viral life cycle. HIV-Tat has also been shown to up-regulate genes associated with translational machinery (de la Fuente et al., 2002).

Nef increases expression of genes regulating membrane lipid biosynthesis genes

To determine the biological significance and pathophysiological relevance of SIV-Nef modulation of genes involved in membrane lipid metabolism, we analyzed the specific functions of the genes in this cluster. The majority of the membrane lipid-metabolism-related genes with increased expression encoded enzymes that catalyze various steps of the membrane lipid

Table 1
RNA processing and transcription regulation related genes modulated by SIV-Nef expression

	Gene/Transcript	Abbreviation	Accession #	Fold increase
1	AU RNA binding protein/enoyl-Coenzyme A hydratase	*AUH*	X79888	1.5
2	Basic leucine zipper and W2 domains 1	*BZW1*	D13630	1.7
3	Cleavage and polyadenylation specific factor 6, 68 kDa	*CPSF6*	X67336	1.8
4	Cleavage stimulation factor, 3′ pre-RNA, subunit 2, 64 kDa	*CSTF2*	NM 001325	1.8
5	Cleavage stimulation factor, 3′ pre-RNA, subunit 2, 77 kDa	*CSTF3*	NM 001326	1.8
6	Crn crooked neck-like 1 (*Drosophila*)	*CRNKL1*	AF255443	1.6
7	CUG triplet repeat RNA binding factor 2	*CUGBP2*	U69546	1.7
8	DEAD (Asp–Glu–Ala–As) box polypeptide 19-like	*DDX19B*	AJ237946	1.8
9	DEAD (Asp–Glu–Ala–As) box polypeptide 1	*DDX1*	NM 004939	1.6
10	DEAD (Asp–Glu–Ala–As) box polypeptide 46	*DDX46*	NM 014829	1.5
11	DEAH (Asp–Glu–Ala–His) box polypeptide 33	*DDX33*	NM 020162	1.8
12	Dicer 1, Dcr-1 homolog (*Drosophila*)	*DICER1*	AB028449	1.6
13	Elongation factor RNA polymerase II-like 3	*ELL3*	NM 025165	1.6
14	Elongation factor RNA polymerase II, 2	*ELL*	NM 006532	2.1
15	Eukaryotic translation initiation factor 1A	*EIF1A*	NM 003758	2.5
16	Eukaryotic translation initiation factor 1A, Y-linked	*EIF1AY*	NM 004681	2.2
17	Eukaryotic translation initiation factor 2B, subunit 1 alpha, 26 kDa	*EIF2B1*	NM 001414	1.5
18	Eukaryotic translation initiation factor 3, subunit 1 alpha, 35 kDa	*EIF3S1*	NM 003758	1.8
19	Eukaryotic translation initiation factor 3, subunit 9 alpha, 116 kDa	*EIF3S9*	U62583	1.6
20	Eukaryotic translation initiation factor 5	*EIF5*	NM 001969	4.7
21	Eukaryotic translation termination factor 1	*ETF1*	NM 004730	2.3
22	Fatty acid binding protein (psoriasis-associated)	*FABP5*	M94856	1.7
23	FUS interacting protein (serine–arginine rich) 1	*FUS*	NM 004960	1.8
24	Heterogeneous nuclear ribonucleoprotein A/B	*HNRPAB*	NM 004499	1.8
25	Heterogeneous nuclear ribonucleoprotein A1	*HNRPA1*	NM 031157	1.9
26	Heterogeneous nuclear ribonucleoprotein C (C1/C2)	*HNRPC*	NM 004500	1.5
27	Huntingtin interacting protein 1	*HIP1*	AF052288	1.8
28	Hypothetical protein DC50	*DC50*	NM 031210	1.6
29	LSM5 homolog, U6 small nuclear RNA associated	*LSM5*	AF182291	1.5
30	Mak3 homolog (*S. cervisiae*)	*MAK3*	NM 025146	1.5
31	Mitochondrial elongation factor, G1	*GFM1*	NM 024996	1.5
32	NTF2-like export factor 1	*NXT1*	AF156957	1.5
33	Nuclear protein UKp68	*ZC3H14*	AF155107	1.9
34	Nuclear RNA export factor 1	*NXF1*	AF112880	2.1
35	Nucleoporin 160 kDa	*NUP160*	D83781	1.6
36	Poly(A) polymerase alpha	*PAPOLA*	X76770	2.5
37	Polymerase (RNA) II (DNA directed) polypeptide I, 14.5 kDa	*POLR21*	NM 006233	1.5
38	RAE1 RNA export 1 homolog (*S. pombe*)	*RAE1*	U84720	1.5
39	Sideroflexin 4	*SFXN4*	XM058406	1.6
40	Splicing factor 3a, subunit 1 120 kDa	*SF3A1*	X85237	1.5
41	Splicing factor proline/glutamine-rich	*SFPQ*	NM 005066	2.0
42	Splicing factor, arginine/serine-rich 1	*SFRS1*	NM 006924	1.9
43	Splicing factor, arginine/serine-rich 10	*SFRS10*	AF057159	1.6
44	Splicing factor, arginine/serine-rich 2	*SFRS2*	NM 003016	1.7
45	Splicing factor, arginine/serine-rich 3	*SFRS3*	NM 003017	2.1
46	Splicing factor, arginine/serine-rich 6	*SFRS6*	NM 006275	2.7
47	Splicing factor, arginine/serine-rich 7, 35 kDa	*SFRS7*	L41887	1.5
48	Splicing factor, arginine/serine-rich, 46 kDa	*SFRS8*	U08377	1.6
49	Synaptotagmin binding, cytoplasmic RNA interacting protein	*SYNCRIP*	NM 006372	2.3
50	Transformer-2-alpha	*HTRA2*	NM 013247	1.7
51	Translation initiation factor IF2	*EIF2*	NM 004090	1.6
52	U2 (RNU2) small nuclear RNA auxiliary factor 2	*U2AF2*	NM 007279	1.6
53	UPF3 regulator of nonsense transcripts homolog A	*UPF3A*	AF318575	1.6
54	Zinc finger protein 265	*ZNF265*	NM203350	1.5

RNA splicing, translation factors, transcription factors and mitochondrial ribosomal proteins related genes were up-regulated in SIV-Nef-expressing T cells.

biosynthetic pathways. These included transferases (1-acylglycerol-3-phosphate *O*-acyltransferase 3, lysophophatidylglycerol acyltransferase 1, sialyltransferase 10, UDP-Gal:beta GLcNAC beta 1,4-galactosyltransferase 5 and UDP-glucose ceramide glucosyltransferase), kinases (ethanolamine kinase), phosphatases (sphingosine-1-phosphate phosphatase 1), CDP-diacylgylcerol synthase and activating proteins (phospholipase A2-activating protein and neutral sphingomyelinase activation associated factor) (Fig. 5). Lysophasphatidic acid acyltransferase catalyzes the acylation of lysophasphatidic acid to phosphatidic acid (Aguado and Campbell, 1998). CDP-diacylglycerol synthase 2 catalyzes the conversion of phosphatidic acid to

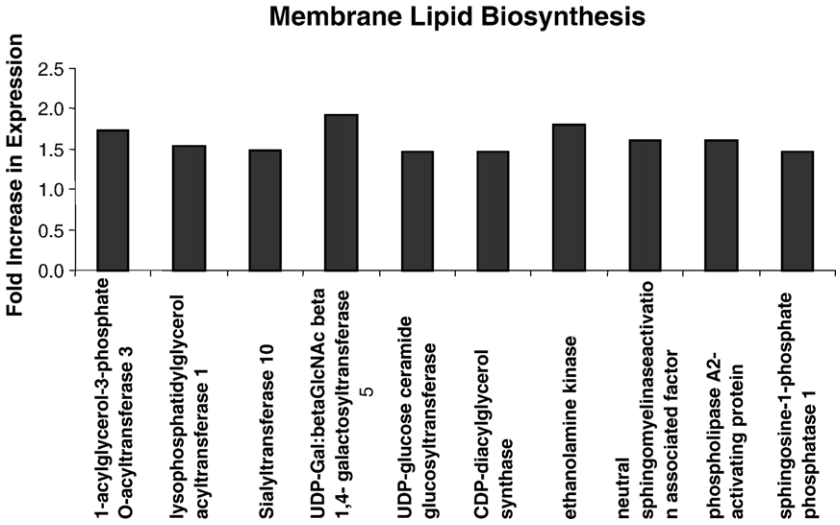

Fig. 5. Membrane lipid-metabolism-related genes modulated by SIV-Nef. Genes encoding enzymes and factors that catalyze the biosynthesis of membrane lipids (glycolipids and phospholipids biosynthesis) were significantly up-regulated in SIV-Nef-expressing Jurkat T cells. Magnitude of up-regulation in fold changes is indicated on the *x* axis.

CDP-diacylglycerol, an important precursor for glycerolipid biosynthesis (Weeks et al., 1997). Ethanolamine kinase catalyzes the first committed step in phosphatidyethanolamine biosynthesis via the CDP-ethanolamine pathway. HIV-1 Nef has recently been shown to enhance the expression of genes encoding membrane lipid and cholesterol biosynthesis and uptake in T cell including ACAT2, DHC24 and CY51A1 that were observed to be up-regulated by SIV-Nef in our study (van't Wout et al., 2005). Overall, these data suggested that SIV-Nef mediated up-regulation of genes encoding enzymes and factors that catalyze the biosynthesis glycolipids and phospholipids, the major components of cell membranes provide yet other mechanisms for role of Nef in the viral life cycle.

Verification of differentially expressed genes

We next sought to verify DNA microarray data by assessing the transcript levels of thirteen genes by utilizing TaqMan real-time RT-PCR (Table 2). Overall, real-time RT-PCR results were in concordance with the DNA microarray results.

Since regulation of RNA transcripts may not correspond to changes in protein levels, we performed immunoblot analysis to determine whether the genes shown to be modulated by SIV-Nef at the transcript level were similarly expressed at the protein level. We examined expression levels of Bcl-2, an anti-apoptotic protein, and cell cycle regulators Cyclin D and Cyclin A which inhibit apoptosis and suppress cell proliferation respectively and modulated by SIV-Nef. Bcl-2 was up-regulated while Cyclin A and D were down-regulated in SIV-Nef-expressing Jurkat cells which were in agreement with the microarray data (Fig. 6).

Discussion

In the present study, we utilized DNA microarray analysis to examine the effect of SIV-Nef on the transcription of genes in $CD4^+$ T cells. Alterations in cellular gene expression in Nef-expressing Jurkat $CD4^+$ T cells are likely to be directly and specifically relevant to the effects of Nef expression in $CD4^+$ T lymphocytes during viral infection. The rationale for our approach is as follows: first, Nef expression levels were found to be similar to those observed in SIV-infected rhesus macaque $CD4^+$ T cells during early stages of infection and CEMx174 cells in vitro (Walk et al., 2001). Second, since Nef is expressed early in the viral life cycle, examining effect of Nef expression 24 h post-induction of Nef expression coincides with the timing of in vivo expression of Nef during early stages of infection. Third, induction of Nef expression in Jurkat $CD4^+$ T cells led to distinct qualitative and quantitative changes in gene expression profiles.

The functional homology between SIV and HIV-1 Nef has been demonstrated in in vitro systems utilizing SHIV nef chimeric viruses in macaque primary lymphocytes and macaque cell lines and the expression of SIV-Nef in human cell line (Alexander et al., 1999; Baur et al., 1994; Sawai et al., 1995; Walk et al., 2001; Yoon et al., 2001). Experimental infection of rhesus macaques with SHIV nef recombinant viral clones has also demonstrated the functional and pathogenic equivalence of HIV-1 and SIV nef in vivo (Kirchhoff et al., 1999; Mandell et al., 1999; Shibata et al., 1997; Sinclair et al., 1997). These studies highlight the biological relevance of utilizing the SIV-Nef/human T-cell line system and SHIV nef-infected rhesus macaque model for studying Nef properties and the role of HIV-1 Nef in HIV pathogenesis.

We focused our analysis on identification of biological themes and functional gene clusters rather than on individual genes. Genes with statistically significant alterations in transcript levels for six overrepresented Gene Ontology functional categories were identified and subjected to further analysis. These included antigen presentation, cell cycle, cell adhesion and RNA processing, protein biosynthesis and degradation and membrane lipid metabolism. Our results are

Table 2
Comparison between DNA microarray analysis and TaqMan real-time RT-PCR data

	Gene	Abbreviation	Accession #	Fold change microarray	Fold change TaqMan real-time RT-PCR
1	Cell division cycle 25C	*CDC25C*	NM 001790	−2.0	−2.5
2	Interferon, alpha-inducible protein (CloneIFI-15K)	*G1P2*	NM 005101	+2.4	+2.3
3	BCL2-associated transcription factor 1	*BCLAF1*	AF249273	+2.3	+2.3
4	TCR gamma variable 9	*TRGV9*	M13231	−2.0	−2.0
5	Death associated transcription factor 1	*DATF1*	NM 022105	−3.1	−1.3
6	Cyclin-dependent kinase 6		AW192700	−2.0	−3.0
7	BCL2/adenovirus E1B 19 kDa interacting protein 3	*BNIP3*	NM 004052	−4.9	−4.5
8	Src family associated phosphoprotein 1	*SCAP1*	NM 003726	−2.1	−2.0
9	Polymerase (RNA) III (DNA directed) polypeptide B	*POLR3B*	NM 018082	+2.8	+2.0
10	Zinc metalloproteinase	*ZMPSTE24*	NM 005857	+2.5	+2.5
11	Polymerase (RNA) I polypeptide C, 30 kDa	*POLR1C*	NM 004875	+2.6	+2.2
12	Polymerase (RNA) III (DNA directed) polypeptide B	*POLR3B*	NM 018082	+2.8	+2.8
13	Src family associated phosphoprotein 1	*SCAP1*	NM 003726	−2.1	−2.0

TaqMan real-time RT PCR analysis was used to validate Nef-mediated changes in genes expression observed using DNA microarray. Real-time RT-PCR results were in concordance with the DNA microarray data.

in agreement with previously reported cellular processes shown by biochemical studies to be modulated by Nef including immune evasion (Le Gall et al., 1998; Schwartz et al., 1996), cell cycle regulation (Ndolo et al., 2002) and lipid metabolism (van't Wout et al., 2005; Zheng et al., 2003).

In our study, Nef-mediated down-regulation of a network of genes encoding members of the antigen presentation machinery is a new finding. Among these, MHC-1 and TCR gene families were significantly suppressed. Interestingly, Nef has been shown to posttranscriptionally down-regulate the cell surface expression of MHC-I molecules, more specifically, HLA-A and HLA-B but not HLA-C or HLA-E (Cohen et al., 1999; Le Gall et al., 1998; Schwartz et al., 1996). Most reports regarding mechanisms of Nef-mediated immune evasion have been inconclusive. Majority of these studies suggest that MHC-I is internalized from the cell surface by endocytosis and relocalized to the *trans*-Golgi networks (TGN) through mechanisms that have yet to be elucidated (Greenberg et al., 1998; Schwartz et al., 1996). In addition, recent data indicate that the adaptor protein AP-1 and PACS are required for the Nef-mediated down-regulation of MHC-I molecules (Piguet et al., 2000; Roeth et al., 2004; Wan et al., 1998). Thus, down-regulation of MHC-1 molecules from the cell surface decreases the recognition and killing of HIV-1-infected cells by virus-specific cytotoxic T lymphocytes, thus promoting immune evasion (Collins et al., 1998). Consistent with previously described Nef-mediated endocytosis of antigen presentations molecules, our results also demonstrated a similar down-regulated transcription of key components of the antigen presentation machinery. In view of these observations, we speculate that Nef mediates immune evasion at both the transcriptional and posttranslational levels. Furthermore, the observation of cellular genes already known to influence immune evasion and antigen recognition such as HLA-E among the down-regulated genes provides additional support, suggesting that some of the differentially expressed genes whose relationships with Nef have not yet been characterized but may have some relevance to Nef functions.

Another category of genes found to be strongly over-represented in our expression profiling was cell cycle regulation. Transcription of a broad range of genes encoding cyclins, cyclin-dependent kinases, cyclin dependent kinase inhibitor and growth factors was repressed. The role of Nef in the suppression of cell cycle progression of $CD4^+$ T cells has previously been reported (Ndolo et al., 2002). Nef has been shown to slow the progression of cells through G_1/S phase transition by down-regulation of Cyclin D1 and Cyclin A. In the present study, suppression of cell-cycle-associated genes in Nef-expressing cells might have occurred through a different mechanism involving cell cycle progression through G_2/M check point and chromatin structure. Nef-mediated down-regulation of CD40− ligand gene transcripts is in agreement with previous studies suggesting that Nef expression suppresses CD40− ligand-dependent immune functions including growth-inhibitory and anti-apoptosis effects through cell cycle blockage and CD154-NF-κB signaling, thus providing yet another mechanism for viral persistence (Qiao et al., 2006; Swingler et al., 2003).Consistent with our data, HIV-Vpr and Tat have been shown to down-regulate CD25C and CD2L6, resulting in the suppression of the progression of cells through

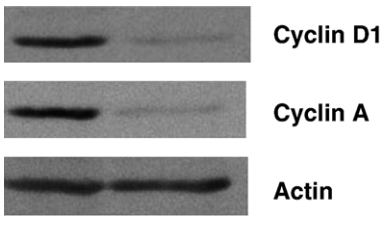

Fig. 6. Immunoblot analysis of Cyclin A and D1 expression in Jurkat and SIV-Nef-expressing cells. Immunoblot analysis was used to determine whether genes modulated by SIV-Nef at the transcriptional level were also regulated at the protein level. The expression of cell cycle regulatory proteins Cyclin D1 and Cyclin A was down-regulated in SIV-Nef-expressing T cells.

G_2/M phase check point (Cujec et al., 1997; de Noronha et al., 2001; Elder et al., 2001; Mahalingam et al., 1998; Meyerson and Harlow, 1994). It has been suggested that Nef-mediated suppression of cell cycle progression through the G_1/S phase and G_2/M phase transition facilitates the completion of the transcription of viral genes and completion of viral life cycle. The Nef-mediated down-regulation of these regulators suggests that SIV-Nef mediated override of the G_2/M phase transition block and inhibition of cell cycle exit, thus facilitating cellular viability and viral persistence. It is worth noting that, in a recent report, ecdysone and its derivatives such as muristerone and ponasterone were shown to induce strong anti-apoptotic effects on mammalian cell including changes in the expression of genes encoding cell cycle regulators contrary to previous reports (Oehme et al., 2006). However, in our study, the observed differences in apoptosis and cell cycle gene expression profiles between ponasterone-A-induced non-Nef-expressing control and ponasterone-A-induced Nef-expressing cells were Nef-mediated. Thus, Nef may enhance the survival of infected cells and viral persistence through different mechanisms. We speculate that the enhanced expression of genes encoding RNA splicing factors, translation factors, mitochondrial ribosomal proteins, transcription factors and other transcription related genes may be explained by the fact that viral infection induces a transcriptional profile that favors viral replication and persistence in the midst of intense antiviral responses. Modulation of these genes may facilitate viral transcription and replication.

Several studies have utilized DNA microarray analysis to investigate transcriptional changes induced by HIV-1 and SIV both in vivo and in vitro (Chun et al., 2003; Geiss et al., 2000; George et al., 2003, 2005; Motomura et al., 2004; Sankaran et al., 2005; van't Wout et al., 2003; Wen et al., 2005). These studies reported increased expression of genes involved in chaperone activity, protein targeting and various aspects of RNA metabolism, including mRNA splicing and processing, transcription, translation and degradation similar to that observed in our study. Four of the above studies specifically examined the effect of HIV Nef expression in different cell types (Kramer-Hammerle et al., 2005; Shaheduzzaman et al., 2002; Simmons et al., 2001; van't Wout et al., 2005).

Recently, van't Wout et al. reported that Nef enhanced the expression of enzymes involved in cholesterol biosynthesis and regulated by the sterol responsive element binding factor-2 (SREBF-2) including cholesterol enzymes (IDI1, FDPS, SQLE, LSS, CYP51, HSD17B7 and DHCR24); one cholesterol regulator (INSIG1); ten sterol enzymes (HMGCS1, HMGCR, MVK, MVD, FDFT1, SC4MOL, NSDHL, EBP, SC5DL and DHCR7) (van't Wout et al., 2005). In the same study, no significant increase in transcript levels was observed for three other genes involved in cholesterol biosynthesis (PMVK, SCAP and INSIG2). Consistent with that study, we found that genes encoding enzymes and factors that catalyze various steps in membrane phospholipid and glycolipid biosynthesis pathways (PLSCR1, DDHD1, ACAT2 and ACSL1) were also up-regulated while three genes SC4MOL, HADHSC and ABCG1 were down-regulated, suggesting that SIV-Nef may broadly enhance membrane lipid biosynthesis and the formation of lipid rafts as previously reported (Zheng et al., 2003). Although the modulated genes may be different, the overall findings with regard to the specific functional cluster, i.e. enhanced expression of genes encoding membrane lipid and cholesterol biosynthesis are essentially similar. Any differences may be attributed to variations in fold change cut-offs and p values used for the selection of differentially expressed genes. More recently, Kramer–Hammerle and colleagues utilized a combination of data and knowledge-driven analysis to characterize Nef-induced gene expression patterns in U251MG cells stably expressing HIV-Nef (Kramer-Hammerle et al., 2005). This approach revealed at least three significantly overrepresented Gene Ontology functional categories including lipid metabolism. Thus, our findings that genes encoding enzymes catalyzing the biosynthesis of membrane lipid in were up-regulated in Nef-expressing Jurkat cells are in agreement with the Kramer-Hammerle et al. study. We speculate that enhanced biosynthesis of membrane lipids may explain the previously reported Nef-mediated enhancement of viral replication and infectivity (Zheng et al., 2001).

The effect of Nef on the transcription of cellular genes in different cell types of epithelial, neural and lymphoid origin was previously investigated using DNA microarray analysis (Kramer-Hammerle et al., 2005; Shaheduzzaman et al., 2002; Simmons et al., 2001; van't Wout et al., 2005). These studies reported that Nef induced a transcription profile that might have favored viral replication (Shaheduzzaman et al., 2002; Simmons et al., 2001). In our study, we found a similar transcriptional profile of differentially regulated genes to that reported in these two studies. However, there are clear differences in the results of the current study presumably due to difference in cell types. Since HeLa cells are not immune cells, they do not exhibit the full range of gene transcripts associated with immune functions. Genes involved in T-cell activation were not significantly modulated in our study. This may be due to the fact that Jurkat T cells are transformed and thus constitutively activated in both Nef-expressing and non-Nef-expressing cells.

In summary, our results demonstrate that Nef expression in Jurkat $CD4^+$ T cells induces a transcriptional profile that promotes viral replication and persistence through sustained immune evasion and survival of T cells. Indeed, HIV-1 has evolved Nef-mediated mechanisms both at the transcriptional and posttranscriptional level to subvert the host immune defense and promote viral persistence during early stages of infection.

Materials and methods

Plasmid constructs

Plasmid pCMV/CD8-Nef, encoding the extracellular and transmembrane domains of human CD8 α chain fused to the SIVmac 239 nef gene, has previously been described (Sawai et al., 1995). Multiple studies have shown that the CD8-Nef

chimera has effects on T cells similar to that induced by constructs expressing wild-type Nef (Baur et al., 1994; Wolf et al., 2001). Inducible SIV-Nef expression plasmid pIND/CD8-Nef was generated by subcloning the CD8-Nef chimera into the ecdysone inducible mammalian expression vector pIND (Invitrogen, Carlsbad, Ca) as previously described (Ndolo et al., 2002). Briefly, the CD8-Nef fragment from pCMV/CD8-Nef was excised using *Hin*dIII and *Xba*I and cloned into the *Hin*dIII–*Xba*I sites of the pIND vector. Expression is under the control of *Drosophila melanogaster* minimal heat shock promoter and the ecdysone and glucocorticoid response element (E/GRE) hybrid promoter. Plasmid pVgRxR (Invitrogen) encodes for the RxR and VgEcR ecdysone receptor subunits and contains a Zeocin selection marker. Two non-Nef-expressing constructs were generated and used as controls, namely: (i) circularized empty pIND vector (without insert); (ii) pIND-CD8-nef plasmid containing SIV-nef gene in reverse orientation subcloned into the pIND vector.

Cell culture, transfection and inducible Nef expression

Jurkat E6-1 T cells and CEMx174 cells (NIH AIDS Research and Reference Reagents Program, Bethesda, MD) were maintained in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine and 100 U/ml penicillin–streptomycin (GIBCO-BRL, Rockville, MD). Jurkat E6-1 T cells were transfected with 20 μg of linearized pIND-CD8-Nef plasmid by electroporation of 5×10^6 cell in 0.5 ml growth media without serum at 250 V and 975 μF using a Gene Pulsor (Bio-Rad, Hercules, CA) and grown overnight. Transfected cells were plated in 96-well plates in growth media containing G418 (1000 μg/ml; GIBCO-BRL) for 14 days. The resulting stable clones were selected by limiting dilution and maintained in the growth media containing 250 μg/ml G418. Cells from stable clones were expanded and transfected by electroporation with linearized pVgRxR using similar conditions as described for pIND-CD8-Nef transfection. Cells were grown overnight and plated in 96-well plates followed by dual selection in the presence of 250 μg/ml G418 and 200 μg/ml Zeocin for 14 days. Clones dual resistant to G418 and Zeocin were expanded and tested for the expression of Nef following induction with ponasterone A, a synthetic analog of ecdysone. Anti-SIV-Nef clone 17.2 antibody (NIH AIDS Research and Reference Reagent Program) was used to detect Nef protein by Western blotting. A ecdysone-inducible Nef-expressing clone, B5 was selected and maintained in RPMI 1640 supplemented with 10% FCS, 100 U/ml penicillin–streptomycin, 250 μg/ml G418 and 100 μg/ml Zeocin. Optimal expression of Nef was attained following induction with 10 μM ponasterone A and 24 h post-induction. Two non-Nef-expressing Jurkat cell lines were generated by stably transfecting Jurkat cells with pVgRxR regulatory plasmid and the control plasmids: (i) circularized empty pIND vector (without insert); (ii) pIND-CD8-nef plasmid containing SIV-*nef* gene in reverse orientation subcloned into the pIND vector.

DNA microarray analysis

RNA extraction, labeling of samples, hybridization to HG-U133A human GeneChips (Affymetrix, Santa Clara, CA, USA), staining and scanning were performed as described in the Affymetrix Expression Analysis Technical Manual. Briefly, total RNA was extracted from: (i) ponasterone-A-induced non-Nef-expressing Jurkat T cells; (ii) ponasterone-A-induced SIV-Nef-expressing Jurkat T cells using RNeasy RNA extraction kit (Qiagen, Valencia, CA, USA) and used as template for cDNA synthesis. Fluorescence intensity values generated from hybridized U133A GeneChips® were subjected to comparative analyses using R statistical software (version 2.01) and BRB Array tools (version 3.3). Changes in the mRNA levels were considered only when: (i) the change in expression levels was statistically significant ($p<0.05$); (ii) the change was equivalent to 1.5-fold change above or below the baseline transcription level in non-Nef-expressing control Jurkat T cells. Biological processes that were statistically overrepresented within each hierarchical subcluster were identified using EASE version 1.0 analysis software (Li and Wong, 2001).

Pathway analysis

Assignment of genes to functional categories was performed through annotation of gene lists using the Affymetrix NetAFFX web interface, the DAVID (http://apps1.niaid.nih.gov/david) annotation tool, and through literature-based classification by hand. Statistically overrepresented (Fisher exact probability score <0.05) biological processes within subclusters were identified using EASE (http://apps1.niaid.nih.gov/david) and Pathway Architect (Stratagene/Ioboin) analysis software. Following elucidation of biologically enriched themes, the microarray data were then re-mined and analyzed with focus on identifying and interpreting all statistically valid changes gene expression associated with the highlighted pathways. In addition, the microarray data were analyzed with GenMapp software (Gladstone Institute: UC, San Francisco) that allows insertion of quantitated transcriptional changes into physiological pathway maps, further enhancing assessment of gene regulation in the context of orchestrated physiological function.

Real-time RT-PCR

The primer probe sets for fourteen genes (a minimum of three genes for each of the following functional categories: immune responses; cell cycle regulation; apoptosis and RNA/protein biosynthesis) were obtained commercially from Applied Biosystems Inc (Foster City, CA). The house keeping genes GAPDH and 18S ribosome were used as internal controls. For each gene, a cycle threshold (Ct) value was obtained at the cycle that produced a detectable fluorescence signal. The 'relative' Ct value of each gene in functional categories was determined by subtracting the Ct value of GAPDH or 18S ribosome from the Ct value of each experimental gene. An average 'relative' Ct value for each gene was calculated from the triplicate repeats of each cell

growth condition. The difference between the average Ct values was used to calculate the fold change for each gene.

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